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Membrane Topology of the Lactococcal Bacteriocin ATP-binding Cassette Transporter Protein LcnC

INVOLVEMENT OF LcnC IN LACTOCOCCIN A MATURATION*

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Many non-lantibiotic bacteriocins of lactic acid bacteria are produced as precursors with N-terminal leader peptides different from those present in preproteins exported by the general *sec*-dependent (type II) secretion pathway. These bacteriocins utilize a dedicated (type I) secretion system for externalization. The secretion apparatus for the lactococcins A, B, and M/N (LcnA, B, and M/N) from *Lactococcus lactis* is composed of the two membrane proteins LcnC and LcnD. LcnC belongs to the ATP-binding cassette transporters, whereas LcnD is a protein with similarities to other accessory proteins of type I secretion systems. This paper shows that the N-terminal part of LcnC is involved in the processing of the precursor of LcnA. By making translational fusions of LcnC to the reporter proteins β-galactosidase (LacZ) and alkaline phosphatase (PhoA*), it was shown that both the N- and C-terminal parts of LcnC are located in the cytoplasm. As the N terminus of LcnC is required for LcnA maturation and is localized in the cytoplasm, we conclude that the processing of the bacteriocin LcnA to its mature form takes place at the cytosolic side of the cytoplasmic membrane.

Several strains of *Lactococcus lactis* secrete antibacterial peptides or proteins with antagonistic activities, the so-called bacteriocins (1, 2). The lactococcins LcnA, B, and M/N are bacteriocins that are synthesized as precursor proteins containing N-terminal leader peptides of the double-glycine type (3). These leaders are removed during maturation. The secretion apparatus for all three bacteriocins consists of the two membrane proteins LcnC and LcnD (4). Similar secretion systems have been described for bacteriocins of other lactic acid bacteria and for colicin V from *Escherichia coli* (5–10).

LcnD is an accessory protein with similarities to other proteins known or believed to be involved in the secretion of various (poly)peptides. They always operate in conjunction with a protein from the family of ATP-binding cassette (ABC)[1] transporters. The accessory proteins of Gram-negative bacteria are proposed to form a family of so-called membrane fusion proteins (11). It is hypothesized that they connect the inner and the outer membranes to facilitate the passage of substrates. CvaA, a member of the membrane fusion protein family, involved in the secretion of colicin V, has been shown to interact with both a cytoplasmic membrane protein (the ABC transporter) and a protein present in the outer membrane (12). The function of these accessory proteins in Gram-positive bacteria is unknown.

LcnC, a protein of 715 amino acids, belongs to the family of ABC transporter proteins present in both prokaryotic and eukaryotic organisms and involved in the import or export of a large variety of substrates such as antibiotics, sugars, amino acids, peptides, and proteins. A typical ABC transporter protein consists of a hydrophobic integral membrane domain and two ATP binding domains. These domains can be either encoded as separate polypeptides or fused into multidomain proteins. The hydrophobic integral membrane domain of the majority of ABC transporter proteins is predicted to consist of two times six transmembrane segments (TMSs) (13). In a number of cases, this topology has been experimentally confirmed (14–17). LcnC contains only one ATP binding domain in its C terminus, whereas the integral membrane domain is, by computer analysis, predicted to consist of four to six TMSs (see below).

LcnC contains an N-terminal domain of approximately 160 amino acids, which shows homology to other ABC transporter proteins involved in the secretion of bacteriocins, and is not present in most other ABC transporter proteins. The N-terminal parts of the ABC transporter proteins involved in the secretion of the bacteriocins pediocin PA-1 and lactococcin G have been shown to process the precursors of the respective bacteriocins into their mature forms. For lactococcin G, this has been shown in *in vitro* experiments (3), whereas for pediocin PA-1 this has been established *in vivo* in the heterologous host *E. coli* (18).

In this paper we show that the N-terminal part of LcnC is involved in the processing of the precursor of LcnA. To examine at which side of the cytoplasmic membrane this processing takes place, we determined the membrane topology of LcnC by making translational fusions of N-terminal parts of LcnC with a modified alkaline phosphatase (PhoA*)[2] lacking its own signal sequence and β-galactosidase (LacZ). The results indicate that four membrane helices of LcnC span the cytoplasmic membrane and that both the N- and C termini are cytoplasmic.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in this study are listed in Table I. *L. lactis* was grown at 30 °C in 2-fold diluted M17 broth (19) supplemented with 0.5% glucose (G1/2M17). For *L. lactis*, chloramphenicol was used at a final concentration of 4 μg/ml. *E. coli* was grown in TY broth (see Ref.

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[1] The abbreviations used are: ABC, ATP-binding cassette; TMS, transmembrane segment; PAGE, polyacrylamide gel electrophoresis; PAA, polyacrylamide; PCR, polymerase chain reaction; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

[2] C. M. Franke, J. Tiemersma, G. Venema, and J. Kok, submitted for publication.

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TABLE I
Bacterial strains and plasmids used in this study
The abbreviations used are: Em, erythromycin; Cm, chloramphenicol; Km, kanamycin; Amp, ampicillin; a.a. amino acid.

Bacterial strain or plasmid	Relevant properties/genotype	Source or Ref.
Bacteria		
L. lactis subsp. *cremoris* MG1363	Plasmid free	50
L. lactis subsp. lactis IL1403	Plasmid free, chromosomally encoded secretion/maturation machinery for LcnA	51
L. lactis subsp. cremoris NZ9000	Plasmid free, MG1363 derivative carrying *nisK* and *nisR* on the chromosome	52
E. coli MC1000	*araD139* Δ *lacX74* Δ *(ara,leu)7697 galU galK strA*	53
*E. coli X*11-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{a}*ZΔM15* Tn*10*(Tet^{r})]	Stratagene
Plasmids		
pMG36C	Cm^{r}, pWV01-based vector containing the strong lactococcal promoter P32	R. Kiewiet[a]
pMG36E	Em^{r}, gene expression vector carrying P32	54
pFUSLC	Cm^{r}, contains *lacZ*	C. M. Franke[b]
pFUSPC	Cm^{r}, contains *phoA**	C. M. Franke[b]
pFUSL32	Cm^{r}, contains *lacZ* translationally fused to the start of *orf32*	C. M. Franke[b]
pFUSP32	Cm^{r}, contains *phoA** translationally fused to the start of *orf32*	C. M. Franke[b]
pLC	Cm^{r}, pFUSLC containing the first 584 codons of *lcnC*	J. Tiemersma[a]
pUC19	Amp^{r}, *E. coli* cloning vector	55
pUK21	Km^{r}, *E. coli* cloning vector	56
pUClcnD	Amp^{r}, pUC19 containing *lcnD*	57
pNZ8048	Cm^{r}, carries the promoter of *nisA*	52
pIL253	Em^{r}, cloning vector	58
pC164	Em^{r}, expressing the first 164 amino acids of LcnC	This study
pNA0	Cm^{r}, *lcnA* and *lciA* under control of P*nisA*	This study
pMGLcnD	Em^{r}, pMG36E derivative with *lcnD* under control of P32	This study
p32LcnD	Amp^{r}, pUC19 derivative with *lcnD* under control of P32	This study
pLC*XXX*	Cm^{r}, pFUSLC carrying parts of *lcnC: XXX* indicates the number of the a.a. residue of LcnC to which LacZ had been fused	This study
pPC*XXX*	Cm^{r}, pFUSPC carrying parts of *lcnC: XXX* indicates the number of the a.a. residue of LcnC to which PhoA* had been fused	This study

[a] From laboratory collection.
[b] Submitted for publication.

20; or 2× TY broth when used for enzyme activity measurements) at 37 °C with aeration. Chloramphenicol, ampicillin, and erythromycin were used at final concentrations of 10, 100, and 100 μg/ml for *E. coli*, respectively. The chromogenic substrates 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and 5-bromo-4-chloro-3-indolylphosphate were used at final concentrations of 5 μg/ml.

Molecular Cloning, Transformation, and Nucleotide Sequencing—DNA techniques were performed essentially as described by Sambrook *et al.* (21). Enzymes were purchased from Boehringer Mannheim GmbH (Mannheim, Germany) and were used according to the instructions of the supplier. DNA was isolated as described before (22) or with the plasmid miniprep isolation kit of Boehringer Mannheim.

Electrotransformation of *L. lactis* was performed as described earlier (23) with modifications according to Venema *et al.* (24). *E. coli* was transformed as described (25). Nucleotide sequences were determined using a Vistra DNA Labstation 625 in combination with a Vistra DNA sequencer 725 (Amersham International, Little Chalfont, UK).

Construction of Plasmids—To overexpress (pre)LcnA in *L. lactis* under control of the *nisA* promoter, *Rca*I and *Hin*dIII restriction enzyme sites were introduced upstream of *lcnA* and downstream of *lciA*, respectively, by PCR on the template pKV4 (26) using the primers CMF60III (5′-AATTCGAATTCTCATGAAAAATCAATTAAATTTTAATATTG-3′) and CMF61 (5′-TCTAGATCTAGAAAGCTTAGAATAGTCGTCCCCACGACATAAT-3′). The PCR product was digested with *Rca*I and *Hin*dIII and cloned in pNZ8048 restricted with *Nco*I and *Hin*dIII, resulting in plasmid pNA0.

Vector pC164 was constructed to express the first 164 amino acids of LcnC. To this end, pPC164 was digested with *Bgl*II and *Asp*718. After filling-in of the sticky ends, the plasmid was self-ligated, giving rise to pIC164. Subsequently, the *Ava*II/*Eco*47-3 fragment of the latter plasmid was transferred to pIL253, resulting in pC164.

To express *lcnD* under control of the lactococcal P32 promoter, the *Sac*I/*Hin*dIII fragment of pUClcnD was cloned in pMG36E, resulting in pMGlcnD. The *Eco*RI/*Hin*dIII fragment of pMGlcnD was transferred to pUC19 to obtain pP32LcnD.

Generation of Directed LacZ and PhoA Fusions*—Based on the working model presented in Fig. 1*A*, primers were designed to introduce *Bgl*II restriction enzyme sites in *lcnC* at various positions flanking the sequences encoding the putative transmembrane segments (TMS). PCRs were performed on pLC as a template, using the various mutagenic primers in combination with primer MvdG1. The sequences of the primers were (*Bgl*II sites are indicated in bold) as follows: MvdG1,

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5′-CCT CGG GAT ATG ATA AG-3′; JT164, 5′-**AGA TCT AGA TCT** TGA CGG GTG ATA ATT GGG-3′; JT194, 5′-**AGA TCT AGA TCT** CTG TCA ATC ATG CTC TGG AGG-3′; JT222, 5′-**AGA TCT AGA TCT** AAG ACC TGT TGG ATA-3′; JT225, 5′-**AGA TCT AGA TCT** GCA AAT TCT AAG ACC TGT TGG -3′; JT246, 5′-**AGA TCT AGA TCT** ATA TAA GAA AGA ATG ACA TC-3′; JT266, 5′-**AGA TCT AGA TCT** GTA ATT TCT CCT GTT CTT CGG-3′; JT277, 5′-**AGA TCT AGA TCT** AAA ATA GAA CTC GCA TCG G-3′; JT303, 5′-**AGA TCT AGA TCT** TTT TGA AGG CCT AAA ATT AG-3′; J T327, 5′-**AGA TCT AGA TCT** GGC GTA AAA ATA ATA ATA AC-3′; JT355, 5′-**AGA TCT AGA TCT** TCA ATC CCA TTG ATA TCT TC-3′; JT375, 5′-**AGA TCT AGA TCT** GCA AAT TCG TAG TCA ATT TTT TG-3′; JT391, 5′-**AGA TCT AGA TCT** TGA ATA GCT TCT GAT TTT TG-3′; JT435, 5′-**AGA TCT AGA TCT** GTA AAG TAA GAA AGC AGG GC-3′; JT478, 5′-**AGA TCT AGA TCT** TGT GAG AGG GAC AGT TC-3′; JT584, 5′-**AGA TCT AGA TCT** GCA TTC TCA TTA GCT CCT AG-3′. After digestion with *Eco*RI and *Bgl*II, the PCR fragments were cloned into either pFUSLC or pFUSPC and verified at the nucleotide sequence level.

Enzyme Activity Assays—Alkaline phosphatase and β-galactosidase assays were performed as described earlier (see Refs. 27 and 28, respectively). Independent cultures were started from three single colonies of each construct. Plasmid stability was checked by plating dilutions of the cultures on plates containing the proper chromogenic substrate prior to the activity measurements. Enzyme assays were performed in triplicate on 2 ml of exponential cultures of *E. coli* and *L. lactis*. All samples used for the enzymatic assays were also subjected to analysis by Western hybridizations.

Protein Electrophoresis and Western Hybridization—After harvesting and washing, the cells were resuspended in the appropriate buffer for either alkaline phosphatase or β-galactosidase activity assays. Parts of the same samples were prepared for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by diluting aliquots of the suspensions 1:1 in 2× sample buffer (29) containing 2.5% SDS and subsequent boiling for 10 min. SDS-PAGE and Tricine/SDS-PAGE were carried out as described earlier (29, 30). Fusion protein samples were run on SDS-5 or 10% PAA gels, whereas samples containing (pre)LcnA were run on either SDS-15% PAA gels or Tricine/SDS-16% PAA gels. Proteins were blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) according to standard protocols (31). LacZ and PhoA* fusion proteins were detected using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Promega, Madison, WI). Polyclonal antibodies against LacZ and PhoA were obtained from 5 Prime → 3 Prime, Inc. (Boulder, CO). Alkaline phosphatase-conjugated mouse anti-rabbit immunoglobulins (Promega) were used as secondary antibodies.

Overexpression of (Pre)LcnA/LciA in L. lactis—Induction of genes under control of the *nisA* promoter were carried out in *L. lactis* NZ9000 containing either pIL253 or pC164. Overnight cultures were diluted 100-fold into fresh medium and grown until an A_{600} between 0.4 and 0.5. Subsequently, the cultures were split in half, and one of these was induced with nisin at a final concentration of 0.5 ng/ml. Both cultures were grown for another 2 h. Purified nisin A was kindly provided by Dr. Oscar Kuipers (NIZO, Ede, The Netherlands).

Overlay Test L—L. lactis cells were disrupted by sonication using the Soniprep 150 (MSE, Crawley, UK) at 8 μm (eight cycles of 15 s with 45-s intervals, all on ice), boiled in 2× sample buffer, and run on Tricine/SDS-16% PAA gels. Gels were overlayered with the indicator strain *L. lactis* IL1403 (18).

RESULTS

Computer Predictions of LcnC Membrane Topology—To obtain a working model for the membrane topology of LcnC, four different computer programs were used that predict the topology of membrane proteins. Due to the various algorithms used by these programs (SOAP (32, 33), Rao and Argos (34), Helixmem (35), and TopPred (36, 37)) all four programs arrived at a different topological model for LcnC. The model obtained with SOAP, contains six transmembrane segments (TMSs) and is shown in Fig. 1*A*. TopPred predicts five of the six TMSs indicated by SOAP (namely I, II, IV, V, and VI), whereas both Rao and Argos (I, II, IV, and V) and Helixmem (II, IV, V, and VI) only predict four.

LcnC Membrane Topology by Protein Fusion Studies—The contradictory results obtained with the various computer programs warranted the analysis of the membrane topology of LcnC by protein fusion studies. N-terminal fusions of LcnC were made with the reporter proteins alkaline phosphatase (PhoA*) and β-galactosidase (LacZ) using the pFUSP/LC plasmid system that we have previously described.[2] The alkaline phosphatase and β-galactosidase activities of exponentially growing cultures of *E. coli* and *L. lactis* carrying the constructed plasmids are presented in Table II. Fig. 1*A* gives an overview of the positions in LcnC to which PhoA* or LacZ were fused.

*Translational Fusions of N-terminal Parts of LcnC with PhoA**—From the data obtained in *E. coli* (Table II), it is clear that at least the N-terminal 164 amino acids of LcnC reside in the cytoplasm; the alkaline phosphatase activity of the fusion protein in which these amino acids are fused to PhoA* (PC164) is low, indicating an intracellular location of the PhoA* moiety. The alkaline phosphatase activity of PC194 is high, indicating that the PhoA* moiety in this chimera is in the periplasm. This strongly suggests the presence of a TMS (TMS I) between the amino acids at positions 164 and 194. The chimeras PC222, PC225, PC246, PC266, and PC277 all have low alkaline phosphatase activities in *E. coli*, indicating that in all these chimeras the PhoA* moiety is intracellular. In accordance with all computer programs tested, these results suggest that TMS II is present and traverses the cytoplasmic membrane, whereas TMS III, only predicted by SOAP, does not exist. The alkaline phosphatase activity of PC303 is high compared with PC277, indicating that TMS IV is present. The same applies to the fusions PC327, PC355, PC375, and PC390, which argues against the existence of TMS V. As the alkaline phosphatase activities of PC478 and PC584 are low, the C-terminal part of LcnC is located intracellularly. This must be facilitated by the presence of TMS VI. However, the high alkaline phosphatase activity of PC435 would argue against the existence of TMS VI.

To examine the expression of the LcnC-PhoA* chimeras, all samples used for the activity assays were subjected to SDS-PAGE and Western hybridizations (Fig. 2, *A* and *B*). As for all chimeras, bands of the expected sizes were detected, lack of activity of certain chimeras cannot be explained by lack of expression of that fusion protein (*e.g.* compare the alkaline phosphatase activities and expression levels of PC194 and PC222 in Table II and Fig. 2*A*, respectively). However, the amount of full-sized fusion protein generally decreased as the size of the chimera increased.

Several LcnC-PhoA* chimeras were expressed in *L. lactis,* and their alkaline phosphatase activities were determined. The results, presented in Table II, are in accordance with those obtained in *E. coli* taking into consideration that, as we have shown previously, alkaline phosphatase activity is high when the PhoA* moiety is in the cytoplasm of *L. lactis*, whereas it is low when PhoA* is extracellularly located.[2] Western analysis showed that the amount of fusion proteins in *L. lactis* with low alkaline phosphatase activity, indicative of an extracellular location of the PhoA* moiety, was much lower than the amount of a chimera with high enzymatic activity (data not shown). This is in accordance with similar topology studies of LcnD (38).

In conclusion, the results obtained with the LcnC-PhoA* fusions indicate that both the N- and C-terminal parts of LcnC are located in the cytoplasm and that four TMSs (I, II, IV, and VI) span the cytoplasmic membrane (Fig. 1*B*).

Translational Fusions of N-terminal Parts of LcnC to LacZ—Genetic instability was observed during construction and maintenance of several of the *lcnC::lacZ* plasmids. Although all cultures were started from a single blue ($LacZ^+$) colony, several of these cultures appeared to consist of mixtures of $LacZ^+$ and $LacZ^-$ cells. Cultures in which this mixed phenotype was observed were discarded. The enzymatic activities in cultures of

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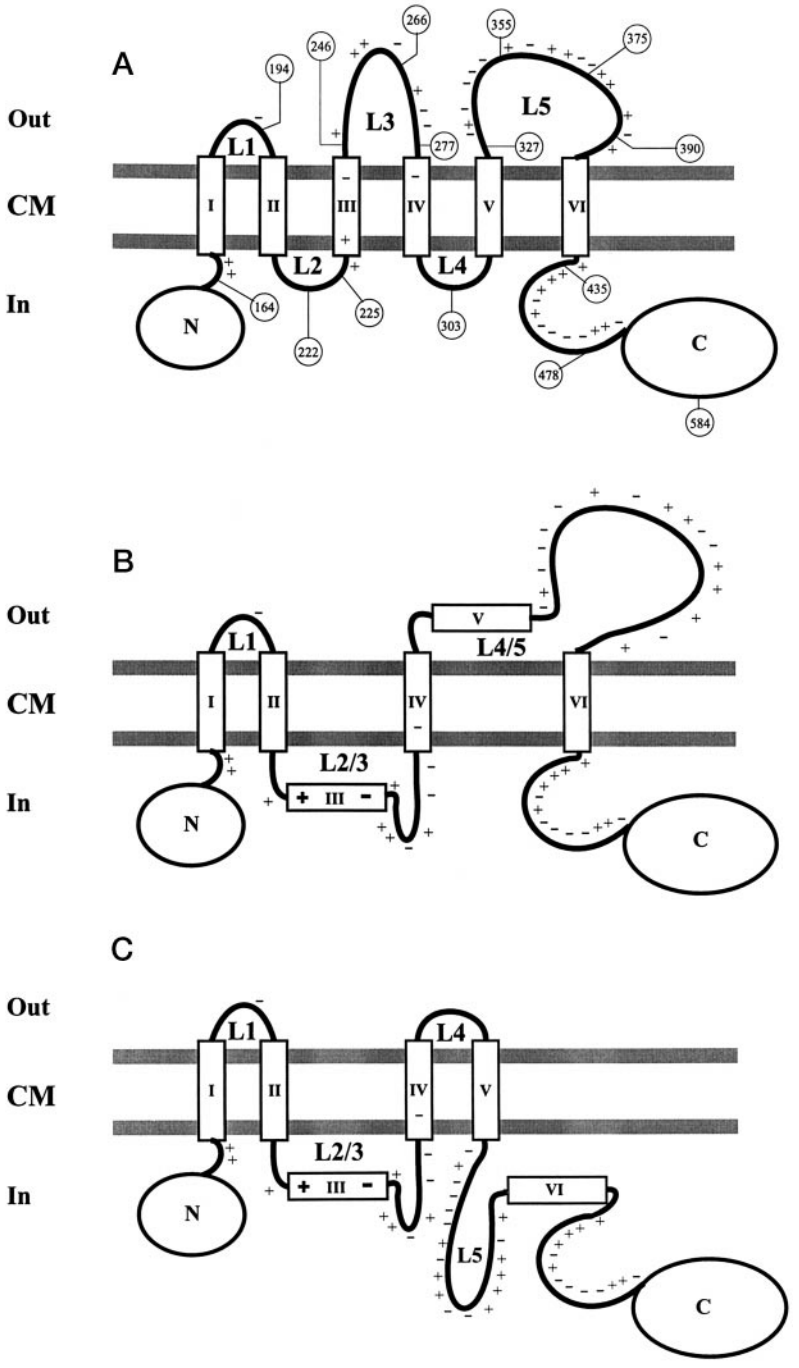

FIG. 1. **Topology models of the *L. lactis* membrane protein LcnC, as predicted by the computer program SOAP (*A*), alkaline phosphatase (*B*), or β-galactosidase (*C*) activities of LcnC-PhoA* and LcnC-LacZ chimeras, respectively.** Positively and negatively charged amino acids are indicated by plus and minus, respectively. Putative transmembrane segments are presented as *open boxes* with *roman numerals*. The *numbered open circles* identify the position of the amino acid residues in LcnC to which the reporter proteins LacZ and PhoA* have been fused. *N,* N-terminal domain (164 amino acids); *C,* C-terminal domain (of 256 amino acids) containing the ATP binding motifs; *L,* loop; *CM,* cytoplasmic membrane.

cells expressing the various LcnC-LacZ fusions are shown in Table II. The location of both the N- and C-terminal domains of LcnC in the cytoplasm of *E. coli* was confirmed by the observation that β-galactosidase activities of the chimeras LC164, LC478, and LC584 were high. The β-galactosidase activity of LC194 was much less than that of LC164, which is in accordance with the presence of TMS I. However, the β-galactosidase activities of those chimeras located in loops 2 and 3 were rather low, whereas on the basis of the PhoA* fusions, the LacZ moiety was expected to reside in the cytoplasm. The β-galactosidase activities of LC222 and LC225 were only slightly higher than that of LC194. The activities of the chimeras in which LacZ had been fused to amino acids of loop 3 in LcnC showed considerable differences; fusions LC246 and LC277 suggest an extracellular location of loop 3, whereas LC266 would rather favor a cytosolic location of loop 3. The low β-galactosidase activity of LC303 indicated that loop 4 is exposed to the periplasm, whereas the high activities of LC355, LC375, and LC390 are indicative for a cytosolic location of loop 5. The low activity of LC327 may be due to the absence of the positive charge of amino acid 331 in the chimera, which may be important for a proper membrane location of loop 5. A similar explanation may apply to the low β-galactosidase activity of LC435, as six positive charges immediately downstream of the fusion point are missing in this chimera.

The differences in the β-galactosidase activities of various LcnC-LacZ chimeras were more pronounced in *L. lactis* (Table II). The activities of LC164, LC435, LC478, and LC584 clearly indicate that the N- and C-terminal domains of LcnC are located intracellularly. Loops 1 and 4 must be exposed at the extracellular side of the cytoplasmic membrane as the β-galactosidase activities of LC194 and LC303 were very low. In con-

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TABLE II

Alkaline phosphatase (PhoA) and β-galactosidase (LacZ) activities in exponentially growing E. coli and L. lactis cells expressing protein chimeras specified by the indicated plasmids*

Plasmid[b]	PhoA* activities[a]		Plasmid[b]	LacZ activities[a]	
	E. coli	*L. lactis*		*E. coli*	*L. lactis*
pMG36C	1 (0)	1 (0)	pMG36C	0 (0)	0 (0)
pFUSPC	1 (1)	1 (0)	pFUSLC	3 (1)	1 (1)
pFUSP32	9 (1)	ND[c]	pFUSL32	144 (11)	ND
pPC164	2 (1)	207 (20)	pLC164	162 (27)	630 (33)
pPC194	100 (9)	1 (0)	pLC194	19 (5)	1 (0)
pPC222	3 (0)	ND	pLC222	30 (4)	ND
pPC225	3 (1)	84 (0)	pLC225	43 (6)	145 (62)
pPC246	1 (0)	ND	pLC246	11 (1)	ND
pPC266	1 (0)	ND	pLC266	38 (2)	ND
pPC277	1 (0)	84 (2)	pLC277	13 (2)	263 (120)
pPC303	30 (4)	2 (0)	pLC303	2 (1)	12 (5)
pPC327	65 (7)	ND	pLC327	7 (1)	ND
pPC355	49 (9)	1 (0)	pLC355	40 (4)	607 (165)
pPC375	46 (0)	ND	pLC375	75 (5)	ND
pPC390	37 (5)	ND	pLC390	51 (24)	ND
pPC435	78 (16)	7 (0)	pLC435	5 (2)	145 (32)
pPC478	14 (4)	11 (1)	pLC478	106 (20)	188 (23)
pPC584	8 (0)	31 (5)	pLC584	72 (39)	93 (25)

[a] Activity units were calculated as $[A_{420} - (1.75 \times A_{550}) \times 1000]/[\text{time (min)} \times A_{600} \times \text{volume of culture assayed (ml)}]$. Standard deviations are given in between parentheses.

[b] Except for pFUSP32 and pMG36C, the numbers in the names of the plasmids correspond to the codons of *lcnC* to which either *'lacZ* or *'phoA** was fused.

[c] ND, not done.

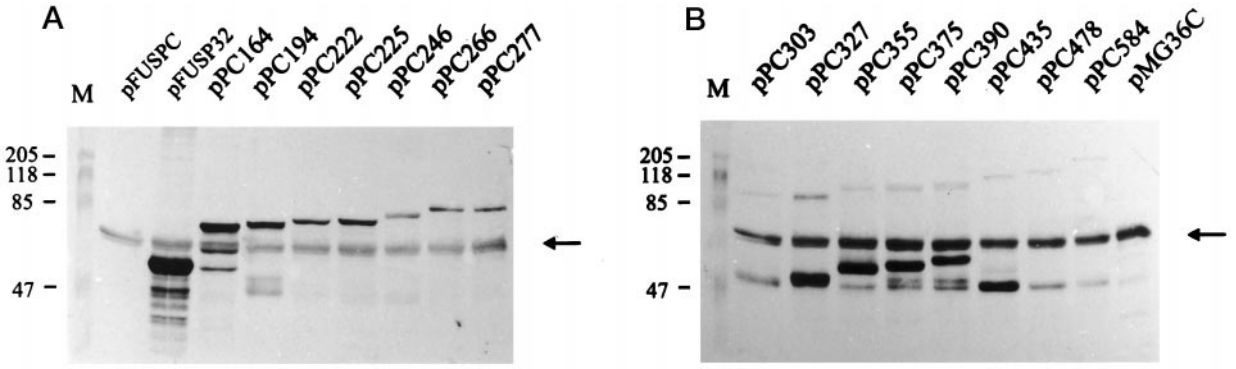

FIG. 2. **Western immunoblots of cell extracts of exponentially growing *E. coli* cultures expressing various LcnC-PhoA* fusion proteins.** The plasmids contained in cells of the individual cultures are indicated *above* each lane. The sizes (in kDa) of the proteins in the protein molecular weight marker in *lane M* are shown in the *left margins*. The LcnC-PhoA* chimeras were detected using anti-PhoA antibodies. The most pronounced slowest migrating band in each lane represents the chimera of the expected size. The band indicated by an *arrow* in the *right margins* corresponds to a protein from the *E. coli* host. *Blot B* has been developed twice as long as *blot A* in order to visualize all the full-sized chimeric proteins.

trast, loops 2, 3 and 5 seem to be cytoplasmic, as judged from the high β-galactosidase activities of LC225, LC277, and LC355.

The expression levels of all LcnC-LacZ chimeras were examined by Western hybridizations (data not shown). Bands of the expected sizes were detected for all chimeras, whereas the amounts of full-sized fusion proteins varied between the LcnC-LacZ chimeras, and proteolytic breakdown was more severe than for the full-sized LcnC-PhoA* chimeras.

In conclusion, the results of the LcnC-LacZ fusions indicate that both the N- and C-terminal part of LcnC are located in the cytoplasm and that four TMSs span the cytoplasmic membrane. Although the data of the LcnC-PhoA* fusions suggest the presence of TMSs I, II, IV, and VI (Fig. 1*B*), the results obtained with the LcnC-LacZ chimeras favor the presence in the cytoplasmic membrane of the TMSs I, II, IV, and V (Fig. 1*C*).

Influence of LcnD on the Membrane Topology of LcnC—To examine whether the membrane topology of LcnC was influenced by the presence of its accessory protein LcnD, pUC19 or pP32LcnD was introduced into the various *E. coli* strains expressing LcnC-LacZ or LcnC-PhoA* fusions. No significant differences were observed in the β-galactosidase or alkaline phosphatase activities, whether LcnD was present or not (data not shown). The enzymatic activities of representative LacZ and PhoA* chimeras in each of the loops were also tested in *L. lactis* IL1403, a strain carrying the genes of LcnC and LcnD homologues on its chromosome (26). The presence of the lactococcin secretion apparatus did not influence the relative enzymatic activities of the various chimeras (data not shown).

PreLcnA Is Processed by the N-terminal Domain of LcnC—To examine whether the N-terminal domain of LcnC, as postulated earlier (3), is involved in the maturation of preLcnA into its mature form, plasmids pC164 and pNA0 were jointly introduced into *L. lactis* NZ9000. A control strain carried pIL253 and pNA0. Production of preLcnA was induced from pNA0, either in the presence (pC164) or absence (pIL253) of the N-terminal 164 amino acids of LcnC. Overexpression of preLcnA was clearly observed in both cases (Fig. 3). No effect of the presence of the N terminus of LcnC on preLcnA overexpression was observed in Coomassie Brilliant Blue-stained SDS-15% PAA gels.

Samples of sonicated cells were subjected to Tricine/SDS-PAGE and were analyzed by overlayer assays (Fig. 4). PreLcnA

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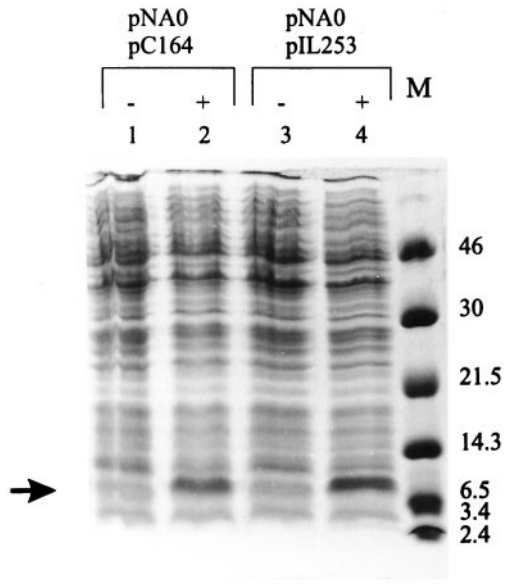

FIG. 3. **Coomassie Brilliant Blue-stained SDS-15% PAA gel used to monitor the overexpression of preLcnA (8 kDa) and LciA (11 kDa) in *L. lactis* NZ9000.** The plasmids present in the cells are indicated *above* the lanes. The sizes (in kDa) of the proteins in the protein molecular mass marker in *lane M* are shown in the *right margin*. The *arrow* indicates the position of the overexpressed proteins in *lanes 2* and *4*. *Lanes 1* and *3,* protein samples of uninduced cultures (−); *lanes 2* and *4,* samples of cultures induced with 0.5 ng/ml nisin A (+).

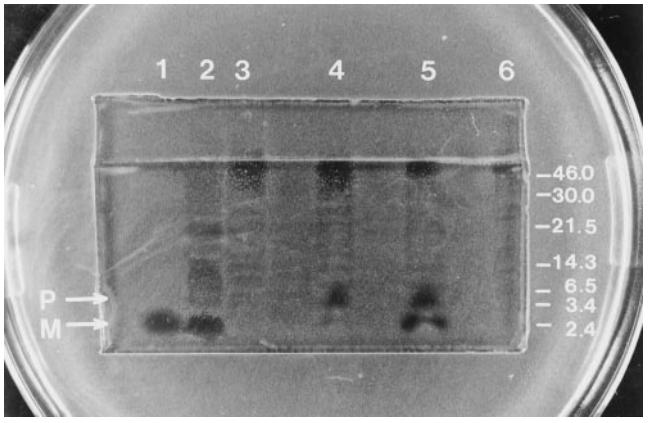

FIG. 4. **Tricine/SDS-16% PAA gel analysis of preLcnA processing.** After electrophoresis, the gel was washed to remove SDS, placed on top of a G1/2M17 agar plate, and overlayered with 10 ml of G1/2M17 top agar seeded with 10 μl of an overnight culture of *L. lactis* IL1403. *Lane 1*, 5 μl of the supernatant of an overnight culture of *L. lactis* IL1403 (pMB553) (49). *Lane 2*, protein molecular weight marker, the sizes of which are shown in the *right margin*. *Lanes 3* and *4*, *L. lactis* NZ9000 (pNA0,pIL253) uninduced and induced with 0.5 ng/ml nisin A, respectively. *Lanes 5* and *6*, *L. lactis* NZ9000 (pNA0,pC164) induced and uninduced, with 0.5 ng/ml nisin A, respectively. *P,* preLcnA; *M,* mature LcnA.

activity was only observed upon nisin induction of the *lcnA* gene present on pNA0. In the presence of the N-terminal 164 amino acids of LcnC, activity of the mature form of LcnA was clearly present, indicating that the processing domain of LcnC resides in the N-terminal part.

DISCUSSION

To elucidate the membrane topology of LcnC, PhoA* and LacZ protein fusions to various N-terminal parts of LcnC were studied. Both the N-terminal domain of approximately 160 amino acid residues and the C-terminal domain of LcnC, containing the ATP binding domain, were shown to be located in the cytoplasm. Therefore, an even number of TMSs should span the cytoplasmic membrane. Indeed, both the PhoA* and LacZ studies indicate that LcnC contains four TMSs in LcnC, but they differ in the exact positioning of these domains. Both reporter enzymes show that TMSs I, II, and IV exist, whereas TMS III, predicted only by the program SOAP, is not present. Ambiguity arises as to whether TMS V or TMS VI is present; although the PhoA* fusions point to the existence of TMS VI, the results obtained with the LacZ chimeras indicate that TMS V traverses the cytoplasmic membrane. These conflicting results may be explained in several ways. First, they may be ascribed to differences in enzymatic activities caused by proteolytic breakdown of the various chimeras. As judged from Western hybridizations (Fig. 2 and data not shown), high levels of protein of the expected size were observed for those chimeras in which either of the reporter proteins had been fused to amino acids within loops 1, 2, and 3 of LcnC (Fig. 1). The levels were significantly lower for those chimeras with fusion points in loops 4 and 5 and for chimeras in which the reporter protein had been fused to LcnC downstream of the predicted TMS VI. This applied in particular to the chimeras in which the reporter protein was fused with amino acid residues 435, 478, or 584 of LcnC, respectively (see Fig. 2*B*). Moreover, for those chimeras with a fusion to amino acid residues 327, 355, 375, or 390, a strong additional protein band was observed that decreased in mobility concomitantly with the corresponding band of the full-length chimeras (Fig. 2*B*). This may be the result of the presence of one or more specific proteinase cleavage sites in loop 5. If certain of these breakdown products are enzymatically active, the resulting background activities might lead to a false topological assignment. If correct, this explanation would favor the model based on PhoA* fusions, as PhoA* first needs to be translocated to the periplasm of *E. coli* to be active. PhoA* retained in the cytoplasm of *E. coli* after proteolytic degradation of an intracellularly located chimera would be inactive due to the absence of disulfide bridges, which are normally formed in the periplasm by DsbA (39) and, therefore, would not lead to a false background activity. LacZ is less reliable in this respect since breakdown products that stay in the cytoplasm could give rise to improper (background) activities.

Alternatively, it is conceivable that LcnC alternates between the two different topological states and that both are, in fact, intermediate stages of LcnC in the bacteriocin secretion process. Our results suggest that loop 5 (situated between the putative TMSs V and VI) of LcnC can either be found at the intracellular or extracellular side of the cytoplasmic membrane. Interestingly, it has been described that the multidrug transporter P-glycoprotein can exist in (at least) two conformations, either with "two times four" or with "two times six" TMSs spanning the cytoplasmic membrane (40, 41). P-glycoprotein is a member of the ABC transporter family, in which two nucleotide-binding sites and 12 putative TMSs are fused together in one polypeptide. Striking similarity is observed between the topological model of the N-terminal part of P-glycoprotein in its two times four conformation and our model of LcnC based on the LcnC-PhoA* fusions.

The fact that the chimeras LC327, LC435, and PC435 were enzymatically active may be due to the circumstance that the reporter protein had been fused immediately downstream of a TMS. It is known that LacZ and PhoA fusions can introduce biases into membrane protein topology analysis (42, 43), especially when positively charged residues in the amino acid sequence downstream of the TMS are absent in the fusion protein, as is the case in these chimeras (44–48).

Apart from these possibilities, the results presented here do not completely exclude the possibility that six membrane hel-

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ices are present in wild-type LcnC as all constructs lack the ATP binding domain, which may be involved in topological changes concurrent with bacteriocin secretion.

To investigate whether loop 5 is able to alternate between a cytoplasmic and extramembranous location, we attempted to raise antibodies against loop five. However, we were not able to overexpress this loop fused to an N-terminal His tag using various systems. Moreover, we were not able to generate reporter protein fusions at the extreme C terminus of LcnC, as *lcnC* appeared to be unclonable in *E. coli* and *L. lactis* under control of P32.

The presence of LcnD in cells producing the various LcnC chimeras had no effect on their enzymatic activities. Apparently, although LcnC and LcnD form a complex in the membrane,[3] LcnD does not influence the membrane topology of these LcnC chimeras.

We have shown here that the N terminus of LcnC is located in the cytoplasm of *L. lactis*. The N-terminal part of LcnC was expected to cleave prelactococcin to the mature active bacteriocin. As prebacteriocin maturation has only been shown *in vitro* or in a heterologous host, we examined whether this could occur *in vivo* in the natural producer organism, in this case *L. lactis*. In the presence of the N-terminal 164 amino acids of LcnC (C164), processing of nisin-induced preLcnA into its mature form was observed in the cytoplasm of *L. lactis*. The tiny amount of mature LcnA present in the absence of C164 may be ascribed to general proteolytic breakdown of the high amounts of prebacteriocin formed inside the cell.

In conclusion, the N-terminal domain of LcnC is involved in the processing of LcnA, a process that takes place at the cytosolic side of the membrane. Based on the results presented in this article on LcnC and those obtained by others with the analogous proteins LagD and PedD (3, 18), we propose to classify a new subfamily of ABC transporters called the ABC-containing Maturation and Secretion Proteins (AMS proteins). Members of this ABC transporter protein subfamily are involved in the secretion of proteinaceous compounds and contain an additional N-terminal domain, involved in the processing of their substrates.

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